Polymorphonuclear Leukocytes, Complement, and *Trichophyton rubrum*

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Trichophyton rubrum can activate complement. In order to assess the role of complement in host defense, fresh human serum was incubated with fungus. Factors were produced which were chemotactic for polymorphonuclear leukocytes (PMNL), but only if complement activation was allowed. This suggests that the chemotactic factor or factors were derived from complement. Incubation of *T. rubrum* with fresh serum did not prevent fungal growth on subsequent culture, but did inhibit incorporation of radiolabeled *N*-acetylglucosoamine. The interaction of PMNL and fungi was studied, and the role of complement as a mediator was assessed. PMNL adhered well to fungi provided that the fungal hyphae had been preincubated with fresh human serum to provide complement opsonins. Opsonized and unopsonized fungi both stimulated a respiratory burst in normal PMNL as measured by chemiluminescence, but the burst was generated much faster with opsonized hyphae. Although hyphae with adherent PMNL subsequently proliferated in culture, the incorporation of *N*-acetylglucosoamine was inhibited 96% when the hyphae were opsonized with fresh serum and then incubated with PMNL. Inhibition was also observed with unopsonized fungi, but to a lesser degree. Varying the ratio of PMNL to hyphae showed that inhibition by PMNL was far more efficient if hyphae were opsonized. In contrast to hyphae, opsonized fungal spores were killed by PMNL so that no growth was observed in subsequent cultures. This killing was not observed if PMNL were omitted or if spores were preincubated with heat-inactivated serum rather than fresh serum. Activation of complement apparently opsonizes the spores so that they can be ingested and killed by viable PMNL. Contents of disrupted PMNL failed to inhibit fungal growth. Complement and PMNL may aid the host in defending itself against infection by dermatophytes. *J Invest Dermatol 86:138–141, 1986*

There are many factors that influence the ability of dermatophytic fungi to infect the skin [1–3]. Recently we found that *Trichophyton rubrum* activated complement, and that this activation occurred primarily by the alternative pathway [4].

Some fungal infections are associated with an accumulation of polymorphonuclear leukocytes (PMNL) beneath the stratum corneum. PMNL are particularly evident when the dermatophyte infects the hair follicles. Kerions are heavily infiltrated by PMNL.

We studied the possible role of complement and PMNL in host defense against the dermatophytic fungus *T. rubrum*.

MATERIALS AND METHODS

Preparation of Dermatophytes *T. rubrum* was isolated from exfoliated scales from a patient with tinea pedis. *T. rubrum* was identified by gross and microscopic examination and by growth characteristics on special media. A small quantity of hyphae was inoculated into 5 ml of Sabouraud liquid broth (modified) and incubated for 1 week at room temperature. The broth culture was shaken vigorously in order to disentangle fungal hyphae and to release fungal spores, and centrifuged at 1700 relative centrifugal force. To resuspend the fungus, 5 ml of phosphate-buffered saline (PBS; 10 mM, pH 7.6) were added back to the inoculum, and pushed briskly back and forth 20 times through 19-gauge and 25-gauge needles. These maneuvers produced a uniform suspension of short fungal hyphae and microaleuriospores. To obtain a suspension of spores alone, the mixture was passed twice through lens paper.

Preparation of PMNL and Serum Fresh human serum was used as a source of complement. Fresh whole blood was allowed to clot at room temperature for 30 min, cooled to 4°C for 45 min, and centrifuged. Serum was stored in 5-ml aliquots at −70°C. Aliquots were depleted of complement activity by heat (56°C for 30 min) (NHS-HI).

PMNL were separated from 30 ml of heparinized fresh human blood by the method of Boyum [5] and washed 3 times in Hanks' balanced salt solution.

Generation of Chemotaxic Factor In order to determine whether *T. rubrum* could generate a chemotactic factor for PMNL by complement activation, normal human serum (NHS) was incubated with hyphae and then tested for its chemotactic activity. *T. rubrum* was washed and resuspended in minimal essential medium (MEM). Next, 20 μl of the fungal suspension were added to 0.5 ml NHS, incubated at 37°C for 1 h, centrifuged, and the supernate tested for chemotactic ability. In order to determine whether complement was responsible for chemotactic activity, fungal suspension was similarly incubated with NHS-HI.

Chemotaxis Assay Chemotaxis was assayed by our modification of the "under agarose" method of Nelson et al [6] and Dahl and Lindroos [7]. The distance from the margin of the well to the leading front of cells was compared with control assays with MEM or NHS-HI (negative controls) or NHS (positive controls) as chemoattractants.

Chemiluminescence In order to determine whether fungi would stimulate a respiratory burst in PMNL, normal PMNL were incubated with opsonized and unopsonized *T. rubrum* and assayed for chemiluminescence according to the method of Nelson et al [8] using luminol as an amplifier. Opsonized zymosan served as positive control, and PBS as negative control. A mixture of dis-

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Abbreviations:
MEM: minimum essential medium
NHS: normal human serum
NHS-HI: NHS inactivated by heating at 56°C for 30 min
PBS: phosphate-buffered saline, 10 mM, pH 7.6
PMNL: polymorphonuclear leukocyte(s)

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rupted fungi was suspended in PBS and separated and washed through a 0.22-μm Millipore filter. Fungi were resuspended in PBS and agitated vigorously with a suspension of 2 mm acid-washed beads to further disrupt the fungal hyphae. The glass beads quickly settled to the bottom, and the fungi were separated from solution by filtration. After drying and weighing, fungi were washed from the filter with PBS, and 0.5 ml of suspension (20 mg/ml) was added to either NHS or NHS-HI 0.5 ml and incubated for 1 h at 37°C. Zymosan was suspended and similarly treated with NHS to serve as a positive control. A 0.3-ml volume of these mixtures was diluted with 0.7 ml PBS in plastic vials along with (5-amino-2,3-dihydro-1,4-phthalazone-dione), dimethylsulfoxide, and normal human PMNL 1.5×10^6. The luminol-amplified chemiluminescence was detected in a Beckman scintillation counter [8]. Assays were done in triplicate. In a follow-up experiment, fungal suspensions were homogenized in a blender to obtain a more uniform suspension of smaller hyphal elements and 5 replicate assays were performed.

Demonstration of PMNL Adherence Fungal suspension 0.1 ml was added to 0.5-ml aliquots of NHS-HI or NHS and incubated for 1 h at 37°C. Polymorphonuclear leukocyte suspension 0.5 ml was added to the mixture of fungus and serum, mixed, and allowed to interact for 1 h at 37°C. Phosphate-buffered saline was added to the mixtures to bring the total volume to 3 ml. A small amount (0.5 ml) of this suspension was passed through a 5.0-μm uniform pore size filter (Millipore, type SM, 5.0 μm) and washed with PBS.

Cells and fungi were fixed in a solution of 2% buffered glutaraldehyde (pH 7.4) for 2 h, stained with hematoxylin and eosin [9], and rendered transparent by immersion in xylene. Each filter was mounted on a glass slide, coverslipped, and examined by ordinary light microscopy.

Viability of Fungus After Interaction with Complement In order to determine whether complement activation could stop fungal replication, fungal elements that had been incubated in NHS were placed in Sabouraud's agar and examined for fungal growth.

In order to determine the effect of complement on spores alone, a suspension of spores was washed twice in PBS, and resuspended in NHS-HI or NHS. After incubation at 37°C for 24 h, spores were examined microscopically for germination. In parallel experiments, germinating spores were incubated with NHS or NHS-HI for 24 h at 37°C. Spores were incubated in liquid Sabouraud's agar for 1 week at 25°C and examined for colony growth.

Viability of Fungus After Opsonization and Interaction with PMNL In order to determine whether PMNL adhering to *T. rubrum* could kill fungi, hyphae were incubated with NHS and NHS-HI and then incubated with PMNL as was done for adherence (described above). Filters were placed onto Sabouraud's solid agar, incubated at 25°C for 1 week, and examined for fungal growth.

Spores are smaller than hyphae and thereby probably easier for PMNL to phagocytose. In order to determine whether PMNL could kill fungal spores, a 0.1-ml suspension containing 100 spores was mixed with NHS 0.5 ml and PMNL (5×10^5 cells/ml of PBS) were added. Controls included: (1) untreated spores; (2) PMNL plus spores and NHS-HI; and (3) spores, NHS, but no PMNL. After incubation at 37°C for 24 h, the contents of each tube were added to 4 ml Sabouraud's broth, incubated, at room temperature for 1 week, and examined for fungal growth.

In order to determine whether PMNL contents could kill fungi, PMNL were concentrated to 5×10^{12} PMNL/ml of PBS and disrupted by freezing and thawing 6 times. Using an inverted microscope, a single germinating spore was aspirated into a specially constructed pipette and transferred into 0.2 ml of the suspension and another spore was transferred to 0.2 ml PBS (control). After 24 h at 37°C, Sabouraud's broth 0.5 cc was added to each sample, incubated at room temperature for 1 week, and examined for fungal growth.

Polymorphonuclear leukocytes or complement might inhibit fungal growth without killing the fungus. In order to investigate this possibility, we studied the ability of complement and PMNL to inhibit the incorporation of radiolabeled N-acetylglucosamine using a modification of the method of Galgiani et al [10]. Ten microliters of the suspension were placed into microwell plates (Falcon). As a control, an identical amount of suspension containing heat-killed fungi (100°C × 120 min) was placed in other wells. Then 100 μl of either NHS or NHS-HI were added to each well and incubated at 37°C in an atmosphere of 5% CO_2 and 95% relative humidity for 2 h. Next, 100 μl of PMNL suspension were added. After 2 h at 37°C, 0.25 μCi (50 μl) of n-acetyl-d-[1-$_3$H]glucosamine (Amersham) was added to each well. After 18 h, 100 μl of PMNL suspension were added to control wells so that all wells contained PMNL, but only half of the wells had contained them during the preceding 20 h when the radiolabel was being incorporated into the fungal cell walls. Immediately 50 μl of sodium deoxycholate 2.5% were added to lyse PMNL, and the contents of each well were passed through a filter (Millipore, type SM, 5.0 μm) to separate hyphae from media. Filters were washed with distilled water and dried. Disintegrations per minute were counted in a liquid scintillation counter.

Since PMNL inhibited incorporation in these experiments whether or not hyphae had been opsonized with NHS, the efficiency of inhibition was examined by varying the numbers of PMNL and thereby varying the ratio of PMNL to fungal hyphae. Concentrations of PMNL tested were 130, 260, 520, 1040, 2080, and 4160 PMNL ml.

Statistical Methods Data were analyzed for statistical significance using a 2-tailed Student *t*-test for independent variables.

RESULTS

T. rubrum grew normally in culture despite incubation of hyphae and spores or spores alone with NHS. Complement was apparently unable to kill the fungus or *permanently* grossly inhibit its subsequent growth in culture.

T. rubrum was able to generate one or more factors from fresh human serum which were chemotactic for PMNL. The mean projected migration of PMNL using fungus-activated serum was 55 mm, whereas the mean migration using MEM was 34 mm in triplicate assays ($p < 0.001$). The mean migration toward zymosan-activated serum was 75 mm and the mean migration toward NHS-HI was 30 mm.

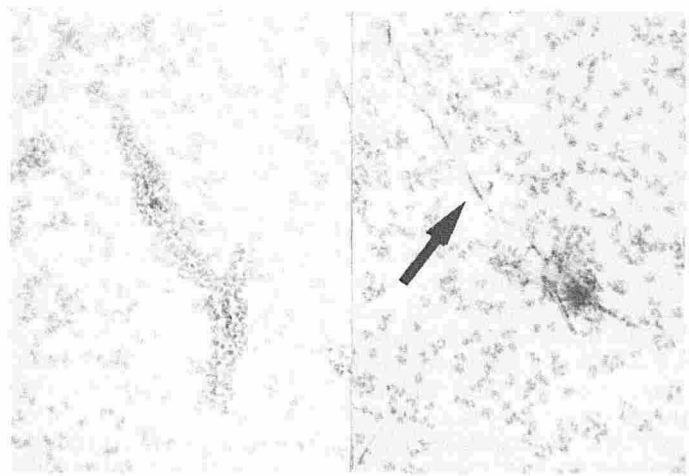

Figure 1. PMNL adhere to fungal hyphae after prior incubation of hyphae with NHS (*a*). This specific adhesion of PMNL to hyphae is not observed if NHS-HI is used instead of NHS as the source of opsonins (*b*). (Original magnification × 120, H&E stain).

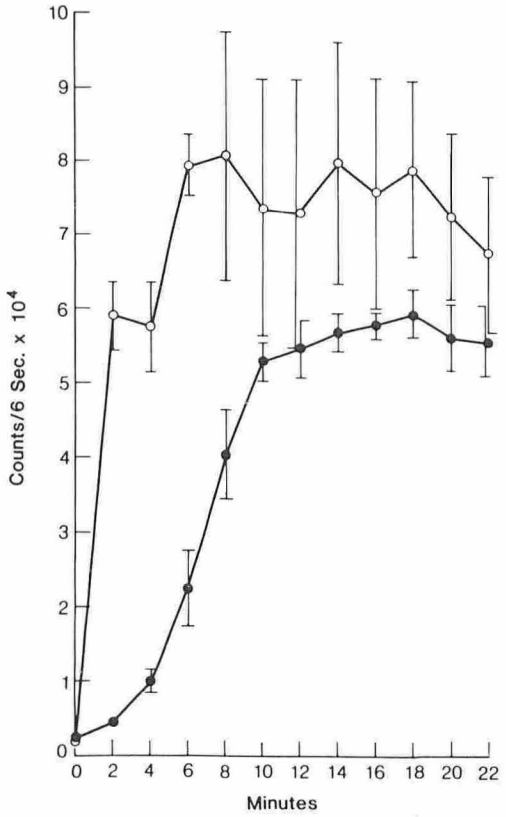

Figure 2. Chemiluminescence response of PMNL by hyphae. The *upper curve* denotes the chemiluminescence after opsonization of hyphae with NHS whereas the *lower curve* denotes the chemiluminescence after similar treatment with NHS-HI.

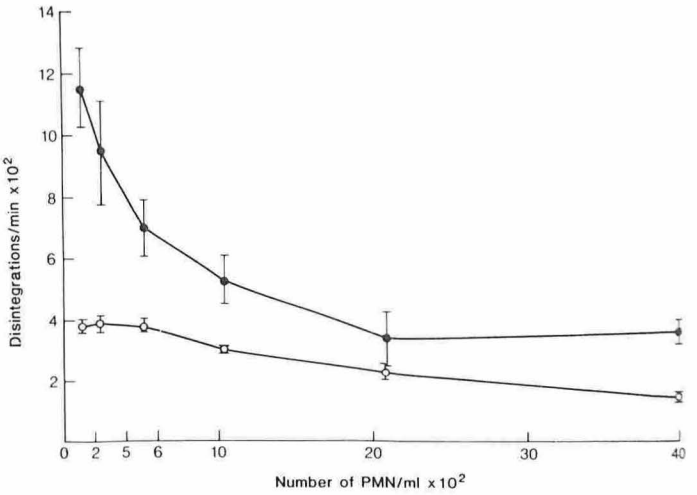

Figure 3. Inhibition of uptake of N-acetylglucosamine by PMNL at various concentrations. The *upper curve* denotes the uptake by hyphae treated with NHS-HI, while the *lower curve* denotes the uptake after treatment with NHS.

PMNL adhered to fungal hyphae in large numbers, provided the fungi had been preincubated with NHS (Fig 1*a*). Adherence was not noted when PMNL were incubated with fungal hyphae pretreated with NHS-HI (Fig 1*b*). When these 2 samples of fungi were placed on agar, there was no difference in growth between fungi coated with PMNL and fungi without them. Abundant and equal growth of fungus was observed.

The contents of disrupted PMNL were not capable of killing the fungi either. The growth of *T. rubrum* incubated with PMNL contents was not prevented. There was abundant growth of fungi after culture of 1 week.

T. rubrum stimulated a respiratory burst in PMNL. This response was augmented if NHS rather than NHS-HI was used to opsonize the fungi, in that the respiratory burst occurred earlier. No chemiluminescence was observed if PMNL were omitted. A weight-equal quantity of opsonized zymosan was a more potent stimulator of the respiratory burst. The maximum chemiluminescence response to opsonized zymosan was 2.24×10^4 after 16 min. By 36 min, the chemiluminescence of PMNL to fungi treated with NHS-HI and NHS was equal. In a follow-up study using a more uniform suspension of smaller hyphae elements, the chemiluminescent response was greater but qualitatively similar (Fig 2). The maximum response for fungi opsonized with NHS was 8.1×10^4 at 8 min, and this was greater than the response to fungi similarly treated with NHS-HI of 4.1×10^4 at that time ($p < 0.001$).

PMNL incubated with opsonized microaleuriospores prevented subsequent growth of *T. rubrum*. If PMNL were excluded or if NHS-HI was used instead of NHS, then no inhibition of growth was observed.

Although NHS and PMNL did not completely prevent subsequent growth in culture, the incorporation of N-acetylglucosamine into fungi was inhibited by NHS when compared with NHS-HI, implying inhibition of growth by complement activation. The mean number of cpm in triplicate assays was 42,260 for heat-inactivated serum and 25,700 for fresh serum ($p < 0.01$). Addition of PMNL after incubation with fresh serum reduced the mean count to 1000, reduction of about 96% from control ($p < 0.001$). The radioactivity associated with heat-killed fungi was approximately 200 cpm in all experiments.

The efficiency by which PMNL inhibit the incorporation of N-acetylglucosamine is depicted in Fig 3. As noted in the experiments described above and also shown in Fig 3, PMNL inhibited uptake whether or not the hyphae were first opsonized with NHS. However, the inhibition was greater if the hyphae were opsonized. Furthermore, the inhibition was far more efficient if the hyphae were opsonized, especially when the ratio of PMNL to hyphae was low.

DISCUSSION

Specific resistance to dermatophyte infection is the province of cell-mediated immunity. Resistance to dermatophytes correlates with skin test reactivity to trichophyton antigens and to the ability to activate lymphocytes in vitro [11,12].

However, host defense against dermatophytic fungi is influenced by other factors. Nonspecific factors that affect the ability of fungus to infect skin and the ability of the host to rid fungus from the skin may include hydration of the skin, anatomic location of the infection, rate of epidermal turnover, and perhaps the chemical and physical nature of the stratum corneum. Serum inhibitory factor is a nonspecific serum factor (unsaturated transferrin) capable of inhibiting the growth of dermatophytic fungi [13].

Could complement also act as an inhibitory factor? Our study suggests that the cytotoxic effects of complement per se are not sufficient to kill the fungus but that complement activation inhibits its growth. Although incubation of *T. rubrum* with fresh serum did not prevent subsequent growth of fungus in culture, complement activation did inhibit the incorporation of N-acetylglucosamine into the cell wall of the fungus.

Complement activation can also mediate the adherence of fungal hyphae. Furthermore, contact of *T. rubrum* with serum generates a factor or factors which are chemotactic for PMNL [14]. Thus complement activation by *T. rubrum* provides a mediator to attract PMNL to the fungus and another to allow PMNL to attach to it.

Contact of *T. rubrum* with PMNL induced a respiratory burst as measured by chemiluminescence. This respiratory burst of PMNL occurred earlier and more vigorously if hyphae were opsonized with NHS.

PMNL are able to kill or inhibit the subsequent growth of spores of *T. rubrum* provided that spores are first treated with NHS. Although fungal hyphae with neutrophils adhering to them grew as well as control fungi when samples were transferred to Sabouraud's agar for culture, spores incubated with NHS and a suspension of PMNL were unable to grow at all. The killing probably requires generation of cytotoxic oxygen intermediates by the PMNL after interaction with fungi [13]. Preformed antimicrobial products of neutrophils might not be as important since fungi grew normally in culture even after incubation of spores with high concentrations of disrupted PMNL contents. However, the killing of spores of *T. rubrum* by PMNL may not be relevant to infections of the skin by this organism since hyphae rather than spores are present within the stratum corneum of infected patients.

N-Acetylglucosamine is incorporated into fungal cell walls as these cell walls are synthesized. Therefore the rate of incorporation of radiolabeled N-acetylglucosamine by fungus serves as a measurement of fungal growth rate. Although complement and PMNL could not kill hyphae, both fresh serum and fresh serum plus PMNL did inhibit fungal growth. Fresh serum alone was also able to inhibit incorporation, but fresh serum and PMNL were capable of inhibiting incorporation to a much greater extent. Possibly this inhibition was due to simple adherence of PMNL to fungi, for example adhering PMNL might block access of fungi to nutrients.

The results of chemiluminescence and incorporation of radiolabeled N-acetylglucosamine are complementary. In both cases, PMNL can interact with unopsonized hyphae but do so better if fungi are pretreated with NHS. Similar interactions have been observed between PMNL and the yeast, *Candida albicans* [13,15].

Ray and Wuepper showed that *C. albicans* could activate complement [16] and that the accumulation of neutrophils subsequent to complement activation was probably due to generation of chemotactic factors of complement such as C5a. They noted that the growth of *Candida* was fostered if animals were pretreated to make them neutropenic [17]. This suggested that neutrophils play a direct role in host defense against *Candida* infection.

We believe that complement activation by *T. rubrum* could mediate the accumulation of PMNL in areas of dermatophyte infection such as kerions, fungal folliculitis, and eczematous plaques. Perhaps the accumulation of PMNL could rid the host of infection, slow down fungal growth to allow its subsequent desquamation, or limit infection to the stratum corneum by eliciting an intense inflammatory response whenever fungal hyphae invade tissue. We speculate that the accumulation of PMNL could also enhance host defense by stimulating epidermal proliferation such that the fungal hyphae are shed as a consequence of an increased rate of desquamation.

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